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ORIGINAL PAPER

Biochar Increases Water Use Efficiency in Eucalypt Plants Under Water and Nutrient Limitation, with Trade-Offs Under Non-limiting Conditions

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Abstract

Soil amendment with biochar is being considered as a strategy for improving available soil water and nutrient content and, thereby, plant performance. Our aim was to investigate whether physiological, biochemical and morphological responses of *Eucalyptus globulus* to biochar amendment were dependent on watering regime. We conducted a randomized, 6-week greenhouse experiment with 5-month old eucalypt rooted cuttings in sandy soil, with the factors: 'biochar application rate' (0% and 4%, ww^{-1}), 'watering regime' (20% and 80% of maximum soil water holding capacity; MWHC) and 'fertilization' (with and without). Increased plant physiological responses to biochar were the most pronounced under water-limited and unfertilized conditions, with a significant increase in leaf water use efficiency (WUE; + 40%), net photosynthetic rate (+ 60%) and plant survival rate (+ 33%), while plant biomass was unchanged. Under water-limited and fertilized conditions, we found no significant biochar effects, except for a small reduction in photochemical and non-photochemical quenching (qP and NPQ, respectively). Under well-watered and fertilized conditions, biochar did not affect leaf WUE or total biomass but reduced the number of branches (− 30%) and photosynthetic rate (− 24%). Finally, under well-watered and unfertilized conditions, biochar was associated with apical leaf deformation, indicating potential micronutrient deficiency, as well as an increase in total soluble sugars and a decrease in stomatal conductance. While the observed benefits suggest that a woody biochar may be advantageous in managing un-irrigated eucalypt plantations, particularly during the planting period, the occurrence of trade-offs urges for long-term studies that account for different dynamic watering regimes, biochar types and application rates, as well as soil–plant-biochar-climate combinations.

Keywords Biochar · Watering regimes · Leaf physiology · Plant growth · Trade-offs · Eucalypt plants

1 Introduction

Biochar is the solid product of thermo-chemical alteration of biomass by pyrolysis. Conceptually, it has been proposed to sustainably improve soil functions (under current and future management) while minimizing potential trade-offs (Verheijen et al. 2015, 2010) and is currently being considered for international policy development, for example, in the IPCC report on Climate Change and Land (IPCC 2019). Biochar characteristics are dependent on biomass and pyrolysis conditions, and in theory, it could be "tailor-made" (Sohi et al. 2009) to improve specific soil natural constraints, without compromising other soil functions (Abiven et al. 2014; Verheijen et al. 2012). In this context, trade-offs need to be accounted for (Jeffery et al. 2015; Tammeorg et al. 2017), to robustly inform on its sustainable use in managing agricultural and forest ecosystems. Recent quantitative reviews agree on a grand mean increase in agricultural crop yield of ca. 10% (Dai et al. 2020; Jeffery et al. 2011; Liu et al. 2013), with greater benefits observed in the less fertile tropical soils (Jeffery et al. 2014, 2017). Liming, improved soil structure,

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plant available water (PAW) and nutrient use efficiency were suggested as the main mechanisms for such benefits to plant yield, upon biochar application (Dai et al. 2020; Edeh et al. 2020; Jeffery et al. 2014, 2017; Razzaghi et al. 2020). Biochar from woody feedstock was shown to be particularly effective in enhancing soil water holding capacity and PAW (Masiello et al. 2015), with contrasting effects depending on biochar age, soil type and irrigation (Aller et al. 2017; Fischer et al. 2019; Razzaghi et al. 2020).

There may also be a potential for biochar to improve the growth of young trees in forest plantations, mainly because these are generally found on un-irrigated land with more natural constraints. An earlier study reported a 13% increase in water use efficiency (WUE) in pine oak in a biochar-amended "low clay" soil (Licht and Smith 2018), which is in line with the estimated 41% biochar-induced increase in biomass of young trees by a global meta-analysis of studies (Thomas and Gale 2015). *Eucalyptus* was not included in the 36 woody plant species that were addressed in that review (Thomas and Gale 2015). While such results are also generally consistent with that reported for various non-woody crops (Gao et al. 2020), they provide an incomplete picture of biochar potential benefits and trade-offs to tree ecophysiological and morphological traits (Kammann and Graber, 2015).

Considering that water limitation has become a worldwide threat to forest plantation sustainability, particularly for well-draining sandy soils in drier regions, it is crucial to implement soil and plant management strategies that can increase plant and forest resilience, under current and future climate scenarios (Allen et al. 2010). Drought effects are plant-specific and depend on stress severity (Ryan 2011). These can manifest through plant morphological, biochemical and/or physiological changes, which often interact and trade-off with plant growth and performance (Ryan 2011).

Due to its fast growth and global economic significance for industry and energy sectors (Booth 2013; Rockwood et al. 2008), the impacts and potential mitigation measures for drought stress in *Eucalyptus* spp. have received much scientific interest. In Portugal, eucalypts are the most common tree species, occupying 26% (812,000 ha) of un-irrigated forest cover (Fernandes et al. 2016; ICNF 2013). To respond to water limitation, *Eucalyptus* spp. plants require to both maximize water uptake and reduce water loss while maintaining their metabolic and physiological needs. Thus, apart from changes in photosynthetic metabolism, drought stress induces a plethora of other adaptations that include (i) decreasing growth rates, total plant and/or root biomass (e.g. Pita and Pardos 2001; Silva et al. 2004); (ii) lowering photosynthetic rates, leaf area and water potential, stomatal conductance and/or internal CO_2 concentration (e.g. Berenguer et al. 2018; Correia et al. 2014a; McKiernan et al. 2016); and (iii) adjusting chlorophyll and carotenoid contents (e.g. Shvaleva et al. 2006) as well as hormonal and metabolite dynamics (e.g. Correia et al. 2016a, b; McKiernan et al. 2016). So far, scarce information exists on biochar's potential to mitigate drought stress responses in woody plants in general, and in eucalypts particularly, or on the range of its impacts on leaf ecophysiological and biochemical traits as well as on plant morphology.

Our main aim was to address these knowledge gaps, by investigating the impact of biochar amendment on the physiological and morphological responses of *Eucalyptus globulus*, as influenced by watering regime. We hypothesized that biochar improves eucalypt plant physiological performance, under water-limited conditions. To test our hypothesis, we conducted a greenhouse pot experiment with rooted cuttings of a representative eucalypt clone, in a sandy soil collected from a eucalypt plantation in central Portugal. We focused on the abiotic mechanism of water availability, where fertilization was included in the experimental design to account for the possible interactions with nutrient availability. We selected the main plant parameters related to the key cellular processes targeted under stress conditions, such as water relations, photosynthetic performance, pigments, oxidative status and carbohydrate content, complemented with morphological traits. Together, they provide a comprehensive picture of both plant primary and secondary metabolisms to allow us to decipher the main potential benefits and trade-offs of biochar application to plant's drought behaviour.

2 Materials and Methods

2.1 Soil and Biochar Characteristics and Pre-treatment

Topsoil sample (15 cm of the Ah horizon) was collected at a *Eucalyptus globulus* Labill. plantation in the inner-dune complex of the coastal zone in Vagos Municipality, central Portugal. The site is approximately 10 km south of the city of Aveiro (44° 42′ N and 80° 42′ W) and has no history of soil contamination. The soil was a Haplic Arenosol (Dystric) (IUSS Working Group WRB 2006) with a sandy texture (99% sand, 1% silt) in a sub-humid meso-Mediterranean climate, with 15 °C mean annual temperature and 950 mm mean annual rainfall. Biochar from mixed wood sieving's feedstock was purchased from Swiss Biochar Gmbh, having been delivered on the 20 of December 2013. The feedstock had been pyrolyzed in a Pyreg 500 III pyrolyzer at a maximum temperature of 620 °C during a 20-min period, resulting in a C content of 80% and a H:C of 0.18. The main physico-chemical soil and biochar properties are listed in Table 1. Bulk density and maximum water holding capacity (MWHC) were measured by weighing and drying (105 °C) three replicates of a known volume. Both soil and biochar

Table 1 Main characteristics of soil, biochar and biochar-amended soil. Abbreviations stand for *MWHC* maximum water holding capacity, *EC* electrical conductivity and *na* not available

	Soil	Biochar	Soil + biochar (4%, w w^{-1})
Bulk density	1.69 (±0.01)	0.17 (±0.01)	1.45 (±0.01)
pH (H_2O)	6.2 (±0.03)	8.1 (±0.04)	7.6
EC (S cm^{-1})	38 (±11)	1,496 (±43)	na
Organic matter (%)	0.8 (±0.06)	88.9 (±2.79)	na
MWHC (%, w w^{-1})	21	na	25
P (mg kg^{-1})	na	217	na
Ca (mg kg^{-1})	na	7,377	na
K (mg kg^{-1})	na	2,029	na
Mg (mg kg^{-1})	na	551	na
B (mg kg^{-1})	na	19	na
Fe (mg kg^{-1})	na	420	na

were air-dried and stored under dark conditions at ambient temperature until use. The pH was measured in water (1:10) for soil 2 weeks after admixing biochar (six replicates) and for biochar without incubation (three replicates). Soil organic matter content was measured by loss on ignition at 550 °C (three replicates). The biochar's (micro) nutrient content was 217 mg kg^{-1} P; 7,377 mg kg^{-1} Ca; 2,029 mg kg^{-1} K; 551 mg kg^{-1} Mg; 19 mg kg^{-1} B; and 420 mg kg^{-1} Fe.

2.2 Experimental Set-Up, Installation and Acclimatization in the Greenhouse

A 5-month-old *Eucalyptus globulus* Labill. rooted cuttings (genotype AL-18) were acquired from ALTRI Forestal's nursery "Viveiros do Furadouro" in Óbidos (Portugal) and transplanted into 1 L treatments pots, with soil and/or biochar, on 7 February 2014. The clone AL-18 is relatively common in this area, with our previous studies indicating good survival rates for this genotype under drought stress (e.g. Correia et al. 2014a). In turn, pot size was selected based on previous studies of similar plant parameters and study durations (e.g. Aller et al. 2017; Heiskanen et al. 2013; Kammann et al. 2011) while considering the available space at the greenhouse. The experiment was designed on a factorial (three factors) basis: biochar amendment (yes/no), watering regime (well-watered and water-limited) and fertilization (yes/no), performing a total of eight treatments at six replicates per treatment (Table 2).

The experiment was conducted in a greenhouse research station of the Siro company (Grupo Leal & Soares, S.A.) located in Mira, Portugal. The greenhouse was not equipped for full temperature control, and considering the unusually cold winter of 2013–2014, a 6-week acclimation period was required before starting the stress induction. During the acclimation period, plants were watered daily to 80% of the MWHC and fertilized once a week with inorganic fertilizer solution (5 mL L^{-1} Complesal 5–8-10 N:P:K), as informed by our previous work (Correia et al 2014a, b). MWHC (aka "field-carrying capacity" or "gravity-drained equilibrium water content" as described by Laird et al. 2010) was determined beforehand in the lab, directly in the pots with perforated bottoms, following the procedure of Verheijen et al. (2019). The mixtures in the pots were wetted gently from the bottom up to saturation, by immersing them into a bucket filled with tap water to avoid aggregates slaking, and then loosely covered with parafilm to reduce evaporation without creating negative pressure in the headspace. The columns were left to drain freely for 24 h, when no further drainage occurred, and weighed. Subsequently, each pot was saturated from the bottom by gentle immersion in a large container filled with the fertilizer solution used during the acclimatization period and allowed to drain freely until reaching 80% of the MWHC.

During the plant experiment, pots were randomized every 2 days. No visual differences among the plants were observed at the end of the 6-week acclimation period and before the start of the stress induction. At the end of the acclimation period, half of the pots with and without biochar were randomly assigned to each of the two water treatments as follows: (1) well-watered, water supplied every day until

Table 2 Summary of treatments. Abbreviations stand for *MWHC* maximum soil water holding capacity

	Biochar (% w w^{-1})	Water level (% MWHC)
Fertilized	0	80
	4 (40 g kg^{-1} soil)	80
	0	20
	4 (40 g kg^{-1} soil)	20
Unfertilized	0	80
	4 (40 g kg^{-1} soil)	80
	0	20
	4 (40 g kg^{-1} soil)	20

soil moisture content (SMC) reached around 80% MWHC; and (2) water-limited, water supplied every day until SMC reached around 20% MWHC (Table 1). Additionally, half of the samples in both watering treatments were either fertilized or not, to exclude confounding biochar effects on plant physiology via the mechanisms of alleviating suppression caused by allelochemicals (e.g. Tian et al. 2007) or soil contaminants (e.g. Gonzaga et al. 2019). At that point, immediately after the acclimatization period, average plant height was 25.4 ± 1.9 cm.

The 6-week stress treatment started on the 21 March 2014. This experimental period was selected based on previous drought stress experiments using this clone (Correia et al. 2014a). Light, temperature, and humidity conditions were semi-controlled and monitored during this period: mean total solar irradiance was 67 W m^{-2}; mean temperature was 20.2 °C, and mean relative humidity was 69.4%. In total, 68 plastic pots were used: 34 pots filled with the collected sandy soil (1,097 g) and the remaining 34 pots with soil amended with 4% (w/w) biochar (797 g soil and 32 g of biochar). On a volumetric basis, the concentration of biochar was 27.5%, corresponding to approximately 90 t ha^{-1} and considering topsoil incorporation (15 cm, assuming 1.5 kg dm^{-3} bulk density), which is towards the higher end of the common range reported by Jeffery et al. (2011). The selected application rate has previously shown to minimize potential trade-offs with other soil functions, namely potential ecotoxicity (Prodana et al. 2019).

At the end of the experiment, the following leaf ecophysiological and biochemical traits, as well as plant survival and morphological parameters were recorded: water potential, leaf gas exchange, chlorophyll *a* fluorescence, plant height, number of leaves and lateral branches, leaf area, dry aerial and root biomass. Leaves were harvested and immediately frozen in liquid nitrogen for further biochemical analysis (photosynthetic pigments content, lipid peroxidation and total soluble sugars).

2.3 Leaf Gas-Exchange Measurements and Shoot Water Potential

Net CO_2 assimilation rate (A, µmol CO_2 m^{-2} s^{-1}), stomatal conductance (gs, mol H_2O m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}) and intercellular CO_2 concentration content (Ci, ppm) were measured in six plants (fully expanded leaves) per treatment, using a portable infrared gas analyser (LCpro-SD, ADC BioScientific Ltd., UK) equipped with a broad leaf chamber. To find out the saturation light intensity, A/PPFD (photosynthetic photon flux density; light response curves of CO_2 assimilation) curves were performed with the following PPFD: 2000, 1500, 1000, 750, 500, 250, 100, 50 and 0 µmol m^{-2} s^{-1}. After A/PPFD data analysis, punctual measurements at saturation light intensity were performed at 750 µmol m^{-2} s^{-1}. The following conditions were maintained inside the chamber during all the measurements: air flux, 200 µmol s^{-1}; block temperature, 25 °C; and ambient atmospheric CO_2 and H_2O concentrations. Data were recorded when the measured parameters were stable (2–6 min). Leaf water use efficiency (WUE) is the ratio of the net rate of photosynthesis (A) and transpiration (E):

$$\mathrm{WUE} = \mathrm{A}/\mathrm{E}$$

Midday shoot water potential was measured with a Scholander-type pressure chamber (PMS Instrument Co., USA) in six plants per treatment at 12 h 30 m (solar time), as described by Correia et al. (2014a).

2.4 Leaf chlorophyll a Fluorescence Analysis and Photosynthetic Pigments

The steady-state modulated chlorophyll *a* fluorescence was determined with a portable fluorometer (Mini-PAM; Walz, Effeltrich, Germany) on the same leaves as used for the gas-exchange measurements (Section 2.5). Light adapted components of chlorophyll fluorescence were measured: steady-state fluorescence (F), maximal fluorescence (F'_m), variable fluorescence F'_v (equivalent to F'_m—F) and quantum yield of PSII photochemistry (ϕ_{PSII}) equivalent to (F'_m—F)/F'_m. Leaves were then dark-adapted for at least 20 min to obtain F_0 (minimum fluorescence), F_m (maximum fluorescence), F_v (variable fluorescence, equivalent to F_m—F_0) and F_v/F_m (maximum quantum yield of PSII photochemistry). NPQ was calculated according to Bilger and Björkman (1990) (NPQ = (F_m – F'_m)/F'_m). Total chlorophyll and carotenoid content were quantified according to Sims and Gamon (2002). Pigments were extracted with acetone/Tris (50 mM) buffer at pH 7.8 (80:20) (v/v) in six independent biological replicates per treatment. After homogenization and centrifugation, supernatants were used to read absorbance at 663, 537, 647 and 470 nm (Thermo Fisher Scientific Spectrophotometer, GENESYS 10-uv S), and pigment content was determined.

2.5 Leaf Lipid Peroxidation and Total Soluble Sugars

Lipid peroxidation was estimated using the method described by Correia et al. (2014a), which measures the amount of leaf MDA (malondialdehyde). Total soluble sugars (TSS) were determined by the anthrone method as described by Irigoyen et al. (1992), by extraction from 50 mg frozen leaves using 80% (v/v) ethanol at 80 °C for 1 h. After centrifugation, the supernatant was mixed with 1.5 mL of anthrone and incubated at 100 °C during 10 min. Absorbance was read at 625 nm, and TSS content was calculated against a D-glucose standard curve.

2.6 Plant Survival, Morphology, Biomass

The dry biomass of the aerial and belowground plant parts was measured after oven-drying at 80 °C until reaching a constant weight. For each plant, all leaves were picked, spread out, photographed and leaf area determined using image processing (ImageJ). Plant survival was determined by visual assessment where only plants without any viable leaves were considered dead. Survival and wilting symptoms were recorded throughout the experiment.

2.7 Statistical Analysis

The effects of biochar amendment, watering regime and fertilization, as well as their interactions on the plant morphological and physiological parameters, were evaluated by means of a three-way ANOVA. When significant interactions between the three factors were observed, subsequent two-way ANOVAs were performed for each level of one factor, using the other two factors in the analysis. The same procedure was followed when interactions between two of the factors were observed, i.e. subsequent one-way ANOVAs were done for one of the factors within the levels of the other factor. If significant differences between the groups of a factor were found following three-way ANOVA, the groups of this factor were considered separately in the subsequent one-way ANOVAs. A Tukey test was used for multiple post hoc comparisons between treatments.

The ANOVAS were only performed if the null hypothesis of homoscedasticity was not rejected by the Levene's test. If, for a certain parameter, homoscedasticity could not be assumed even following standard data transformations, the effects of each combination of the different factors (treatments) were tested separately using the non-parametric Kruskal–Wallis (KW) test. Whenever the differences between the treatments were significant ($\alpha < 0.05$) following the KW test, the Dunnett's test was used for multiple post hoc comparisons between the treatments.

Outliers were removed based on the modified Thompson-Tau for each treatment. If a specific plant was an outlier for two or more 'independent' (i.e. not directly related) ecophysiology variables, it was removed for all ecophysiology variables. The same was done for the plant morphology variables. All statistical analyses were performed using SPSS v. 22.

3 Results

Biochar effects on selected soil parameters are shown in Table 1. Biochar reduced soil bulk density by 14% and increased soil water holding capacity (w/w) by 19%. Further, soil pH also increased upon biochar application, from 6.2 to 7.6 (Table 1).

There were significant interactions between biochar and watering regime ($B \times W$), and to a lesser extent, between biochar and fertilization ($B \times F$) or between the three factors ($B \times W \times F$; Tables 3–5). The interplay $B \times W$ had the largest effects on the measured parameters, both at the level of leaf physiology (E, gs, A, NPQ, carotenoid and sugar content; Tables 3–4) as well as plant morphology (leaf area and dry aboveground biomass; Table 5). In turn, the interaction B × F was only significant for a few leaf physiological parameters (Ci, A and WUE; Table 3), whereas $B \times W \times F$ influenced leaf WUE, anthocyanin and sugar contents.

Plant responses to biochar are described in sub-sections 3.1, 3.2, 3.3 and 3.4 for each treatment combination, following the same order provided in Tables 4 to 5. Table 3 shows the results of selected plant ecophysiological traits, while Table 4 and Table 5 show, respectively, effects on leaf biochemical traits and plant survival and morphology (recorded at the end of the experiment).

3.1 Plant Responses to Biochar Under Well-Watered and Fertilized Conditions

Biochar amendment decreased photosynthesis by 24% under well-watered and fertilized conditions. Whereas no other significant effects were observed on plant ecophysiology or biochemistry (Tables 3 and 4), there was a significant reduction (by up to 30%) in the number of branches in response to biochar (Table 5).

3.2 Plant Responses to Biochar Under Well-Watered and Unfertilized Conditions

For this treatment, biochar amendment resulted in nearly double the content of total soluble sugars (TSS; Table 4) in leaves and a significant decrease in gs and Ci (Table 3). There were no observed morphological differences, except for apical deformation in plants in biochar-amended soil (Fig. 1).

3.3 Plant Responses to Biochar Under Water-Limited and Fertilized Conditions

In this treatment, biochar only had significant effects on plant ecophysiological parameters, having caused a reduction in qP and NPQ (Table 3).

3.4 Plant Responses to Biochar Under Water-Limited and Unfertilized Conditions

Biochar had the greatest effects on plant physiological, biochemical and morphological parameters under water and nutrient limitation. Plants in biochar-amended soil showed

Table 3 Mean and standard deviation values for the leaf ecophysiological traits for each biochar treatment and the corresponding control (no biochar), under the study watering and fertilization regimes. Abbreviations stand for *E* transpiration rate, *gs* stomatal conductance, *A* net CO_2 assimilation rate, *WUE* water use efficiency, *qP* photochemical quenching and *NPQ* non-photochemical quenching, *ϕPSII* quantum yield of PSII photochemistry and *Fv/Fm* maximum quantum yield of PSII photochemistry. For each individual parameter and interaction of factors (*B* biochar, *W* watering regime and *F* fertilization regime), significant values are indicated in bold, with the corresponding notation $*p<0.05$, $**p<0.01$, $***p<0.005$

	Well-watered				Water-limited				Interactions		
	Fertilized		Not fertilized		Fertilized		Not fertilized				
	Biochar	No biochar	Biochar	No biochar	Biochar	No biochar	Biochar	No biochar	$B\times W$	$B\times F$	$B\times W\times F$
Ci (ppm)	276 ± 24	271 ± 15	**278** ± 7*	**298** ± 14	247 ± 20	227 ± 15	**243** ± 10*	**280** ± 26	0.892	**0.003*****	0.212
E (mmolH_2O m^{-2} s^{-1})	3.23 ± 0.51	3.76 ± 0.41	3.48 ± 0.13	3.78 ± 0.33	2.04 ± 0.14	1.99 ± 0.29	2.73 ± 0.18	2.44 ± 0.62	**0.036***	0.395	0.997
gs (molH_2O m^{-2} s^{-1})	0.40 ± 0.15	0.60 ± 0.22	**0.44** ± 0.04*	**0.67** ± 0.17	0.16 ± 0.02	0.16 ± 0.04	0.26 ± 0.03	0.22 ± 0.10	**0.015***	0.959	0.713
A (μmolCO_2 m^{-2} s^{-1})	**13.3** ± 1.42*	**17.5** ± 1.00	14.4 ± 1.01	14.2 ± 0.77	10.1 ± 0.99	11.7 ± 1.61	**15.2** ± 1.03*	**9.5** ± 1.48	**< 0.001*****	**< 0.001*****	0.096
WUE (mmol mol^{-1})	4.20 ± 0.80	4.68 ± 0.46	4.15 ± 0.31	3.80 ± 0.46	4.95 ± 0.75	5.88 ± 0.47	**5.59** ± 0.38*	**3.99** ± 0.69	0.331	**< 0.001*****	**0.045***
Water potential (MPa)	− 0.70 ± 0.40	− 0.59 ± 0.08	− 0.62 ± 0.08	− 0.54 ± 0.10	− 1.45 ± 0.38	− 1.32 ± 0.30	− 1.05 ± 0.20	− 1.36 ± 0.13	0.275	0.163	0.221
NPQ	6.76 ± 1.09	7.61 ± 1.49	7.20 ± 1.43	5.95 ± 1.75	**4.74** ± 1.80*	**7.60** ± 1.53	**6.51** ± 0.44*	**8.79** ± 1.11	**0.004*****	0.142	0.396
qP	2.42 ± 0.20	2.72 ± 0.35	2.69 ± 0.35	2.76 ± 0.24	**2.02** ± 0.27*	**2.73** ± 0.32	**2.47** ± 0.13*	**2.80** ± 0.24	0.052	0.070	0.655
Φ PSII	0.69 ± 0.04	0.66 ± 0.10	0.69 ± 0.07	0.66 ± 0.07	0.54 ± 0.17	0.69 ± 0.06	0.69 ± 0.02	0.73 ± 0.02	0.310	0.273	0.354
Fv/Fm	0.84 ± 0.005	0.83 ± 0.008	0.83 ± 0.014	0.79 ± 0.031	0.85 ± 0.008	0.83 ± 0.007	0.83 ± 0.009	0.84 ± 0.002	0.169	0.416	0.126

Table 4 Mean and standard deviation values of the leaf biochemical traits for each biochar treatment and the corresponding control (no biochar), under the study watering and fertilization regimes. Abbreviations stand for *MDA* malondialdehyde and *TSS* total soluble sugars. For each individual parameter and interaction of factors (*B* biochar, *W* watering regime and *F* fertilization regime), significant values are indicated in bold, with the corresponding notation $*p<0.05$, $**p<0.01$, $***p<0.005$

	Well-watered				Water-limited				Interactions		
	Fertilized		Not fertilized		Fertilized		Not fertilized				
	Biochar	No biochar	Biochar	No biochar	Biochar	No biochar	Biochar	No biochar	$B\times W$	$B\times F$	$B\times W\times F$
Anthocyanins (μmol $g^{-1}FW^{-1}$)	0.42 ± 0.05	0.76 ± 0.11	0.47 ± 0.06	0.48 ± 0.06	0.78 ± 0.14	0.56 ± 0.06	0.39 ± 0.12	0.65 ± 0.12	0.364	0.8549	**0.024***
Carotenoids (μmol $g^{-1}FW^{-1}$)	0.62 ± 0.18	0.48 ± 0.41	0.47 ± 0.14	0.43 ± 0.06	0.69 ± 0.27	0.66 ± 0.25	0.51 ± 0.16	0.67 ± 0.46	**0.040***	0.080	0.608
MDA (eq $g^{-1}FW^{-1}$)	0.69 ± 0.10	0.69 ± 0.13	0.54 ± 0.05	0.48 ± 0.06	0.64 ± 0.05	0.70 ± 0.15	0.66 ± 0.04	0.80 ± 0.25	0.113	0.856	0.362
TSS (mg $g^{-1}FW^{-1}$)	10.9 ± 8.63	10.7 ± 3.21	**25.6** ± 3.68*	**10.9** ± 4.16	40.4 ± 7.81	43.0 ± 9.35	**18.7** ± 9.88*	**57.2** ± 16.90	**0.006****	0.95	**0.002*****

Table 5 Mean and standard deviation values of the whole plant morphological traits for each biochar treatment and the corresponding control (no biochar), under the study watering and fertilization regimes. Abbreviations stand for *ABG* aboveground biomass. For each individual parameter and interaction of factors (*B* biochar, *W* watering regime and *F* fertilization regime), significant values are indicated in bold, with the corresponding notation *$p<0.05$, **$p<0.01$, ***$p<0.005$

	Well-watered				Water-limited				Interactions		
	Fertilized		Not fertilized		Fertilized		Not fertilized				
	Biochar	No biochar	Biochar	No biochar	Biochar	No biochar	Biochar	No biochar	$B\times W$	$B\times F$	$B\times W\times F$
Survival (%)	100	100	100	100	100	100	100	67			
Height (cm)	51.3 ± 2.22	53.4 ± 1.47	52.0 ± 0.71	53.7 ± 1.15	44.0 ± 1.22	44.5 ± 4.44	43.6 ± 4.29	43.6 ± 2.10	0.395	0.502	0.470
Branches (n°)	**6.40** ± 1.34*	**9.20** ± 0.84	7.17 ± 2.64	8.60 ± 1.82	5.60 ± 1.52	5.40 ± 1.82	5.17 ± 2.93	5.40 ± 1.82	0.246	0.812	0.455
Leaf area (cm^2)	819 ± 115	891 ± 105	704 ± 96	844 ± 168	395 ± 68	276 ± 107	340 ± 112	270 ± 75	**0.000*****	0.431	0.322
Root biomass (g)	2.48 ± 0.36	2.88 ± 0.71	2.74 ± 0.18	2.16 ± 0.85	1.44 ± 0.15	1.75 ± 0.81	1.61 ± 0.27	1.89 ± 0.67	0.255	0.197	0.231
ABG biomass (g)	36.9 ± 4.71	41.4 ± 5.44	31.5 ± 3.45	39.6 ± 6.55	19.1 ± 2.28	15.2 ± 3.57	16.5 ± 4.15	13.4 ± 3.53	**0.001*****	0.353	0.767
Root:shoot	0.25 ± 0.04	0.25 ± 0.05	0.29 ± 0.06	0.20 ± 0.07	0.26 ± 0.03	0.38 ± 0.17	**0.28** ± 0.03*	**0.80** ± 0.11	0.324	0.361	0.334

increased photosynthetic rates (A) and WUE (Table 3), although Ci, qP and NPQ were significantly reduced (Table 3). Biochar amendment also decreased leaf TSS content by > 30%, compared to the no-biochar treatment (Table 4). Despite having had a positive effect on plant survival (100% survival in biochar-amended soil, compared to 33% in the absence of biochar), it more than halved the root:shoot ratio in surviving plants (Table 5).

4 Discussion

Since the strongest observed interaction was between biochar and watering regime, with the most pronounced effects under water limitation, we firstly discuss the results for water-limited and subsequently, for well-watered conditions. The weaker observed interactions between biochar and fertilization are integrated into the respective discussion for each watering regime, for simplification.

The most consequential biochar effect on plant responses under water limitation was the improved leaf WUE (by 40%) in unfertilised conditions, which is likely to be linked to the increased photosynthesis (A; by 60%). This disagrees, to some extent, with that reported by Licht and Smith (2018), where the 13% increase in WUE in pine oak in response to biochar was attributed to the decreased photosynthetic activity and stomatal conductance under irrigation deficit. However, the possibility of biochar eliciting a plant stress response under water limitation, as suggested by Licht and Smith (2018), could also be applied to the present study. In fact, we observed reduced photochemical quenching (NPQ and qP), TSS content (67%) and root:shoot ratio (65%) in this treatment, despite the mechanisms involved in photosystem efficiency, energy dissipation, oxidative and drought stress not having been impaired (Liu et al. 2011; Sánchez-Rodríguez et al. 2010). While a more detailed discussion into trade-offs is provided in the paragraphs below, this indicates that either biochar did not elicit a stress response under drought, or that any such response was small, when weighted against the main mechanisms by which biochar is thought to improve leaf WUE (Gao et al. 2020): (i) liming (soil pH increase), (ii) biochar C content and/or (iii) biochar K content. While watering regime was not considered as a possible contributing mechanism in their meta-analysis (Gao et al. 2020), the grand mean effects of leaf WUE increase for the categories used by Gao et al. (2020) with relevance to the present study were (i) 52% (soil pH = < 7), (ii) not significant (> 80% biochar C) and (iii) 12% (< 1% biochar K). This allows us to infer that the observed increase in WUE (40%) upon biochar amendment under unfertilised conditions was likely to be primarily caused by a liming mechanism and secondarily by a K fertilization mechanism. The fact that improved leaf WUE could be partially attributed to

Fig. 1 *E. globulus* plant growing in sandy soil under well-watered conditions: plant presenting apical deformation in the biochar treatment (**A**) and without apical deformation in the biochar-fertilizer treatment (**B**)

nutrient inputs via biochar amendment could also explain why biochar had no significant effect on A under fertilised conditions. Nutrient input via biochar, particularly in respect to K (2,000 mg kg^{-1} in our biochar), is thought to play a role in drought tolerance strategies (Kammann and Graber 2015), including in *E. grandis* (Battie-Laclau et al. 2016), but it was not the case for pine oak (Licht and Smith 2018). Further, while biochar K or fertilizer inputs have formerly been found to contribute to increasing WUE in non-woody crops (Faloye et al. 2019; Gao et al. 2020), in the present study, fertilization did not contribute to significantly improve WUE in biochar-amended soil. On the contrary, it suggests that biochar may have, at least partly, limited uptake of the supplied nutrients, through mechanisms that may be influenced by specific biochar-soil–plant interactions and biochar ageing (Kammann and Graber 2015).

Increased leaf WUE and net photosynthetic rate may also explain, at least partly, the 100% survival rate observed under drought and unfertilised conditions, compared to 67% for the same treatment without biochar. Together, they indicate a potential biochar contribution to improve *E. globulus* performance and long-term survival strategies towards drought tolerance, although for *E. grandis* and *E. urophylla x E. grandis*, no changes in survival were observed upon biochar application (Farias et al. 2019; Rockwood et al. 2019). Considering the vast eucalypt planted areas and their attributed economic value, particularly in regions where PAW is a limiting factor to plant performance, such benefits anticipate a potentially valuable application of biochar in agroforestry, reforestation and/or ecosystem restoration under water and nutrient limitation, similarly to that demonstrated for autochthonous tree species (Farias et al. 2016). Nonetheless, comparisons with the relevant literature are limited by the small number of available studies using woody plant species and leaf WUE measurements (which are mainly employed in field studies; Licht and Smith 2018; Medrano et al. 2015). Further, other mechanisms linked to drought tolerance in eucalypts may have also played a role in the observed responses (e.g. phytohormonal pathways; Kammann and Graber 2015). It is also likely that soil texture, biochar type, application rate and age may be additional influencing factors for WUE responses in *E. globulus*, similarly to that found in other crops (Aller et al. 2017; Gray et al. 2014), and therefore, our results in a sandy soil should be carefully extrapolated to other soil types. The impact of fluctuating soil moisture contents (e.g. via rainfall) might also impact on biochar-driven changes in WUE and should be considered in future studies.

Under conditions where water supply was not a limiting factor to plant growth and performance, the main observation was the occurrence of trade-offs on leaf physiology and plant morphology in response to biochar amendment. In the unfertilized treatment, we observed a reduction in Ci (by 34%) and in gs (by 10%) as well as apical deformations, while in the fertilized treatment, there was a decrease in A (by 30%) and in the number of branches (by 24%). Despite physiological responses of woody plants to biochar remaining poorly addressed, similar trends have been reported for non-woody crops, as influenced by watering regime (at biochar application rates < 20 t ha^{-1}; e.g. Langeroodi et al. 2019). While changes in PAW are often linked to changes in plant nutrient dynamics (Kammann and Graber 2015), micronutrient deficiency, rather than N limitation, may have contributed to such responses. Biochar commonly enhances soil nutrient bioavailability by increasing soil pH and CEC of acidic soils (Jeffery et al. 2017; Kloss et al. 2015). However, soil pH was already at the optimum range for *E. globulus* (Thomson et al. 1996) before biochar application; thus, a liming effect (i.e. over-liming) could have reduced the solubility and subsequently, bioavailability of micronutrients, resulting in apical deformation under well-watered conditions. Specifically, boron deficiency may have potentially contributed to the observed symptoms of metabolic disorder (rolled and malformed leaves and stem dieback), similarly to that observed in several woody species (Moretti et al. 2012), including in *E. globulus* (Dell and Malajczuk 1994). However, future studies would need to be designed to prove this.

Reduced micronutrient bioavailability due to (over)liming could also explain, in part, the lack of significant changes in plant aboveground biomass and height in biochar amended soil (without water limitation). Based on the results of Rockwood et al. (2019) for *E. urophylla x E. grandi*, as well as the reported increased height of non-woody crops (e.g. Aller et al. 2017; Faloye et al. 2019), biochar application was expected to increase plant growth. However, the only observed plant morphological change to biochar was a reduction in the number of branches, which may reflect a stress response. Other stress responses in *E. globulus*, such as that caused by apical insect defoliation, are known to manifest in changes in branch biomass (Quentin 2010). In turn, Batool et al. (2015) observed a 50% reduction in leaf area and plant biomass, but did not report micronutrient deficiency symptoms, although those are likely to have occurred at the studied pH interval (9.1 at 1% biochar and 8.8 at 3% biochar). Inconsistent observations with respect to plant physiological and morphological parameters may be explained by differences in crop types, soil structure, biochar feedstock and application rate having been used, all of which may be additional determining factors in morphological responses to biochar or its interactions with fertilization and irrigation. Despite the dynamics of (micro)nutrients with soil pH and plant uptake being beyond this study's aim, they may vary for different soil-biochar-plant combinations and require further assessment.

In summary, understanding how, and through which mechanisms, woody plant physiology responds to water limitation in a specific soil-biochar system remains unclear (Ayles et al. 2015), as most of the available literature focuses on biomass and yield-related parameters (Jeffery et al. 2011; Thomas and Gale 2015). Specifically, the occurrence of trade-offs urges the need for long-term studies that account for dynamic watering regimes (natural rainfall), as well as different biochar types (from structural and nutritional feedstocks), application rates, soil–plant-biochar-climate combinations and pot sizes, acknowledging that undesired side effects are as informative as the desired effects in building a sustainable biochar system (Verheijen et al. 2012, 2015, 2019).

5 Conclusions

There was a significant interaction between biochar amendment, watering regime and fertilization in *Eucalyptus globulus* growing in a representative sandy textured soil. Under water-limited and unfertilized conditions, pinewood chips biochar led to increased leaf water use efficiency, net photosynthetic rate and plant survival. These observations validate the hypothesis that soil amendment with a woody biochar can reduce the impacts of water and nutrient limitation in this plant species, being potentially relevant in managing un-irrigated eucalypt plantations during dry periods in the planting season. In turn, observed physiological and morphological trade-offs upon biochar amendment were the most pronounced under well-watered conditions, indicating that benefits might be offset under irrigation or during wet periods. The occurrence of trade-offs urges for further research to confirm causal mechanisms and outcomes under field conditions with dynamic weather conditions.

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Author Contribution F. Verheijen and G. Pinto were the main contributors to the study conception and design. All authors contributed to the experimental work, data collection and treatment. Statistical analysis was mainly performed by A. Vasques. F. Verheijen, A.C. Bastos and G. Pinto prepared and revised the manuscript and performed the quality checks and the proof reading. All authors read, commented on and approved the revised version of the manuscript.

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Data Availability The authors declare transparency for all data presented in the manuscript.

Declarations

Conflict of Interest The authors declare no competing interests.

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